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Biochemical identification of base and phosphate contacts between Fis and a high affinity DNA binding site

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SUMMARY

Fis is a nucleoid-associated protein in *E. coli* and other bacteria that stimulates certain site-specific DNA recombination events, alters DNA topology, and serves as a global gene regulator. DNA binding is central to the functions of Fis and involves a helix-turn-helix DNA binding motif located in the carboxy-terminal region. Specific DNA binding is observed at a number of sites exhibiting poorly related sequences. Such interactions require four critical base pairs positioned –7, –3, +3, and +7 nucleotides relative to the central nucleotide of a 15 bp core-binding site. To further understand how Fis interacts with DNA, we identified the positions of fourteen DNA phosphates (based on ethylation interference assays) that are required for Fis binding. These are the 5' phosphates of the nucleotides at positions –8, –7, –6, +1, +2, +3, and +4 relative to the central nucleotide on both DNA strands. Another five phosphates located in the flanking regions from positions +10 through +14 can serve as additional contact sites. Using a combination of biochemical approaches and various mutant Fis proteins, we probed possible interactions between several key Fis residues and DNA bases or phosphates within a high-affinity binding site. We provide evidence in support of interactions between the R85 Fis residue and a highly conserved guanine at position –7, and between T87 and the critical base pairs at –3 and +3. In addition, we present evidence in support of interactions between N84 and the phosphate 5' to the base at +4, between R89 and the –7 phosphate, between T87 and the +3 and +4 phosphates, and between K90 and the +3 phosphate. This work provides functional evidence for some of the most critical interactions between Fis and DNA required for a high binding affinity and demonstrates the large contribution made by numerous phosphates to the stability of the Fis-DNA complex.

Keywords

Fis; nucleoid-associated protein; DNA binding; ethylation interference; missing base contact

INTRODUCTION

Fis (factor for inversion stimulation) is a nucleoid-associated protein (NAP) found in *Escherichia coli* and other enteric bacteria.^{1; 2; 3; 4} Like other NAPs, Fis affects the topology of the chromosome, functions as a global regulator of several hundred genes, and participates in several different site-specific DNA recombination events.^{5; 6; 7; 8; 9; 10} Crystal structures

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revealed that Fis is a homodimer composed of two identical 98 amino acids subunits.^{11; 12; 13; 14} Each Fis subunit contains a flexible or disordered N-terminal region followed by a bundle of four alpha helices with the two C-terminal helices forming a helix-turn-helix DNA binding motif (HTH) (Fig. 1a). Although Fis binds DNA non-specifically at very high concentrations, many of its functions require that it binds and bends specific DNA targets. However, unlike many of the other members of the HTH family of DNA binding proteins, Fis interacts specifically with poorly related DNA sequences. This is reflected in the degenerate consensus sequences constructed from alignments of known Fis binding sites.^{4; 15; 16; 17}

Significant progress has been made in uncovering the Fis and DNA determinants required for high-affinity DNA binding. Studies based on random mutagenesis and single alanine replacements identified 10 Fis residues within or near the HTH motif that were required for DNA binding.^{17; 18; 19; 20} Heat denaturation studies indicated that the overall structural stability of Fis was not significantly affected by the alanine substitutions.²⁰ Single alanine replacements of R85, T87, and K90 (Fig. 1b) severely disrupted interactions with several different specific DNA binding sequences, indicating that these residues are consistently required for specific Fis-DNA interactions.²⁰ Alanine replacements of residues N84, R89, and K91 strongly reduced binding to some but not all DNA binding sequences examined, suggesting that these residues also played important roles in at least a subset of Fis binding sequences.

On the other hand, a recent comprehensive nucleotide replacement study demonstrated that there are four critical base pairs in a Fis binding site that are required for high Fis-binding affinities: $-7G\bullet C$, $-3R\bullet Y$, $+3Y\bullet R$, and $+7 C\bullet G$, where R represents a purine, Y represents a pyrimidine, and the numbers represent the nucleotide distances upstream or downstream of the central base pair of a Fis binding site.²¹ Results from DNA missing base assays more specifically showed that the highly conserved guanine in the base pairs positioned at -7 and $+7$ and the thymine within the $-3A\bullet T$ and $+3 T\bullet A$ base pairs played prominent roles in Fis binding. In addition, AT-rich sequences near the center of the Fis site and immediately flanking the 15 bp core-binding site contributed to the binding affinity and to the DNA flexibility required for Fis-induced DNA bending. These observations suggested that specific Fis-DNA interactions rely on a remarkably small number of base contacts and must therefore rely on a large number of phosphate contacts along the DNA backbone.

In this work, we made use of ethylation interference assays to identify fourteen phosphates along the core region of a Fis binding site that are involved in Fis-DNA interactions. Studies with a DNA binding-deficient Fis mutant also demonstrated the ability of this protein to interact with five additional phosphates in the flanking regions. We further provide biochemical evidence in support of the involvement of N84, R89, T87, and K90 in interacting with 6 of the phosphates in the core DNA binding region, of R85 in interacting with the $-7G$ base on either DNA strand, and of T87 in interacting with the base pairs at -3 and $+3$. The results of this work present a dramatic picture of the prominent role of phosphate contacts in Fis-DNA interactions and increases our understanding of the molecular details of critical Fis-DNA contacts made in a high-affinity Fis binding site.

RESULTS

DNA backbone phosphates involved in Fis-DNA interactions

In order to identify which DNA phosphates are involved in Fis-DNA interactions, we performed ethylation interference assays on a 43 bp DNA fragment carrying a high-affinity Fis binding sequence ($A_{-8}G_{-7}C_{-6}T_{-5}C_{-4}A_{-3}A_{-2}A_{-1}T_0T_{+1}T_{+2}T_{+3}G_{+4}A_{+5}G_{+6}C_{+7}T_{+8}$).²¹ Ethylation of phosphates that participate in protein-DNA interactions generally interferes with complex formation. Therefore, the pool of ethylated DNA fragments retaining their ability to

bind Fis will be depleted of such ethylated phosphates. This results in a reduction in the intensity of the DNA cleavage signals corresponding to these phosphates in the bound compared to unbound DNA. The results showed that ethylation of phosphates 5' to the nucleotides -8A, +1T, +2T, +3T and +4G in each DNA strand strongly interfered with Fis binding, and ethylation of phosphates 5' to -7G and -6C on each strand moderately interfered with binding (Fig. 2a). Thus, a large number of phosphates (14 in total) are involved in Fis binding to the core region of a high-affinity site and are likely sites of Fis contact. These phosphates are clustered in two regions of each DNA strand (Fig. 2b). One region is located around the junction of the 15 bp core Fis binding sequence and the flanking DNA (-8P, -7P, -6P). The other region is located on the other half of the Fis binding site near the center of the sequence (+1P, +2P, +3P, +4P). The positions of these phosphates are all on the same face of the double helical DNA, consistent with their roles as Fis contact sites (Fig. 2c).

Base contacts in Fis-DNA complexes

Having identified the prominent DNA phosphates (Fig. 2), bases,²¹ and Fis residues (Fig. 1)²⁰ required for high-affinity Fis binding, we set out to examine how some of the Fis residues might interact with DNA. We hypothesized that one or more of the five most important Fis residues required for DNA binding (i.e. R85, T87, R89, K90, and K91)²⁰ would likely contact one of the critical base pairs in a Fis binding site (e.g. -7G•C, -3A•T, +3T•A, and +7C•G).²¹ To investigate this, we made use of a "loss of contact" assay, which is based on the rationale that a nucleoprotein complex formed with a Fis mutant that is missing a side chain required for a specific base contact will be largely unaffected by the presence or absence of the matching base on the DNA. Hence, no further loss in the binding affinity should occur when the matching base pair contact is also absent. On the other hand, considerably greater destabilization of a mutant Fis-DNA complex would result from a base pair replacement if the base pair makes a different or additional contribution to the binding affinity.

The wild-type (WT) Fis binds to a high-affinity site (A₋₈G₋₇C₋₆T₋₅C₋₄A₋₃A₋₂A₋₁T₀T₊₁T₊₂T₊₃G₊₄A₊₅G₊₆C₊₇T₊₈) with a dissociation constant (K_d) of about 1 nM Fis in gel electrophoretic mobility shift assays (GEMSA). However, as previously observed,²¹ binding of WT Fis to a DNA site carrying either a -3C/+3G or a -7T/+7A nucleotide combination was severely reduced, requiring μ M Fis concentrations for significant DNA binding (Fig. 3). About 20% of the DNA carrying the -3C/+3G site was bound in the presence of 1.3 μ M WT Fis; about 26% of the DNA carrying the -7T/+7A site was bound in the presence of 3.3 μ M WT Fis. Consistent with previous observations,^{18; 20} the T87A mutant Fis showed a severe reduction in binding to a high-affinity site compared to WT Fis (Fig. 3). In the presence of 1.3 μ M T87A Fis, about 18% of DNA carrying the high-affinity site was bound. Similar binding activity of T87A Fis occurred with the DNA site variant carrying the -3C/+3G nucleotide combination occurred with comparable Fis concentrations (Fig. 3). In the presence of 1.3 μ M T87A Fis, about 21% of DNA carrying the -3C/+3G site was bound. This indicated that the replacement of the original -3A/+3T with the -3C/+3C nucleotide combination, which strongly reduced Fis binding, had no appreciable effect on the binding by T87A Fis. This suggests that the base pairs at positions -3 and +3 are functionally connected to the role of T87 and are therefore possible matching contacts. In contrast, when the DNA binding site variant carrying the -7T/+7A nucleotide combination was used in these assays, no binding was detected with as much as 4.4 μ M T87A Fis, indicating a severe reduction in binding to this site compared to the original high-affinity Fis binding site or to the sequence carrying the nucleotide replacements at -3 and +3 positions. Binding by T87A to a DNA site carrying the -7T/+7A nucleotide replacements was also noticeably reduced compared to the binding by WT Fis to this same site (Fig. 3). These observations suggest that the T87 Fis residue and the base pairs at positions -7 and +7 positions make different contributions to the binding affinity and are not matching contact sites.

Compared to WT Fis, the mutants R89A, K90A, and K91A all showed a considerable reduction in binding to the high-affinity Fis site (Fig. 3). Whereas 50% binding to this DNA occurred with about 1.2 nM WT Fis, roughly 55% binding occurred with 40 nM R89A Fis. In addition, only about 3.5% and 21% DNA binding was observed with 220 nM K90A and 220 nM K91A Fis, respectively. However, when DNA sites carrying the $-3C/+3G$ or $-7T/+7A$ nucleotide replacements were used, a substantially greater reduction in DNA binding was observed by these same three Fis mutants since no binding activity was detected with as much as 2.2 μ M mutant Fis (Fig. 3). The results suggest that the R89, K90, and K91 Fis residues interact with sites other than the base pairs at positions $-3/+3$ and $-7/+7$.

The R85A Fis mutant did not give rise to a discernible bound complex with the high-affinity Fis binding site with as much as 4.4 μ M protein (not shown) and was therefore not examined in a similar fashion by GEMSA. R85 is the first residue in the recognition helix of the Fis HTH region (Fig. 1). Residues at equivalent positions in the HTH motifs of other prokaryotic DNA binding proteins are often found forming a base contact on the corresponding DNA binding sites, thereby contributing to the specificity of binding.^{22; 23; 24; 25; 26} Fis-DNA docking models^{15; 27; 28} place the R85 side chain extending in the direction of the $-7/+7$ positions on both halves of a DNA binding site (Table 2) and previous experiments using randomly debased DNA showed that the guanine base of the base pairs at positions -7 and $+7$ (but not the complementary C bases) were essential for Fis binding.²¹ Thus, we made use of a missing-base assay to determine whether or not the presence of the $-7G$ base is essential in DNA complexes with R85A Fis mutants. However, because R85A Fis does not form stable DNA complexes at any concentration examined, we used instead Fis heterodimers composed of R85A and WT Fis subunits. This is possible because of the rapid Fis subunit exchange that is observed to occur in solution.²⁹ By combining in solution R85A Fis with WT Fis in a 33:1 ratio, most of the R85A Fis subunits will associate with other R85A subunits, but such dimers will not contribute significantly to DNA binding at concentrations below 4 μ M. On the other hand, most of the WT Fis subunits will dimerize with R85A subunits during subunit exchange so that essentially all the binding activity will be conducted by R85A/WT Fis heterodimers. Our rationale was prompted from previous observations showing that a double base pair replacement at positions -7 and $+7$ resulted in >1000 -fold reduction in binding but a single bp replacement at one of these two positions resulted in about a 3.3-fold reduction in binding, suggesting that Fis contacts with $-7G$ at only one of the two half sites is sufficient to sustain a relatively strong binding affinity.²¹ Thus, we anticipated that WT/R85A Fis heterodimers, having only one R85 side chain available per dimer to interact with DNA, would similarly exhibit a moderate loss in binding, thereby allowing us to examine its DNA binding requirement. Indeed, the WT/R85A Fis heterodimer preparation was seen to lower the DNA binding activity by about 3.7-fold compared to the a concentration of WT Fis that is present in the heterodimer mixture, as expected if substantial WT/R85A heterodimers were being formed (not shown). However, a similar addition of excess R85A to an oxidized S30C Fis mutant, which forms an intersubunit disulfide bond and displays very similar DNA binding as WT Fis,³⁰ does not affect the DNA binding by compared to that of S30C Fis alone, as expected if subunit exchange with S30C Fis is prevented.

As previously observed,²¹ DNA complexes formed between WT Fis and randomly depurinated DNA were depleted of DNA molecules lacking $-8A$, $-7G$, $-2A$, or $-1A$ in the Fis binding site, indicating that the presence of these purines is required for Fis binding (Fig. 4a). Base substitution analysis showed that, of these bases, $-7G$ played the most critical role in Fis binding.²¹ However, complexes formed between the R85A/WT heterodimers and depurinated DNA showed an increased tolerance to the removal of $-7G$ compared to the same complexes formed with WT Fis (Fig. 4a). In contrast, the relative intensities of all other cleavage signals in complexes formed with the R85A/WT Fis preparation were comparable to or lower than those observed in the complex with WT Fis. The increased tolerance for the removal of $-7G$

(and only -7G) in DNA fragments bound to the R85A/WT heterodimers suggests that the -7G base is a matching contact site for the R85 Fis residue (Fig. 4c). At best, an attenuated -7G signal was expected from this assay since a bound heterodimer could tolerate the removal of -7G from one but not both DNA strands simultaneously per bound complex. Any small amount of binding activity potentially caused by residual levels of WT Fis homodimers in the protein mixture would not contribute to an increase in the -7G cleavage signal in these assays.

Depyrimidinated DNA complexes formed with WT Fis were considerably depleted of DNA molecules lacking -2T and -3T (Fig. 4b), suggesting that these bases contributed to the binding of WT Fis. However, depyrimidinated DNA complexes formed with T87A Fis tolerated the removal of both +2T and +3T, suggesting that the T87 residue in WT Fis is somehow linked to the roles of these two bases. These observations are consistent with those of the previous GEMSA results indicating that the base pairs at -3 and +3 positions are required for WT Fis binding but not for T87A Fis binding (Fig. 3). On the other hand, T87A is less tolerant than WT Fis to the removal of +8T, -5T, -9T, -11T, and -19T, but preferentially binds to DNA fragments lacking -16C, -17C, and -18C (Fig. 4b). This suggests that the loss of DNA contact caused by the replacement of threonine 87 with alanine imposes a strong dependence on Fis interactions with the DNA flanking the core binding sequence. Such interactions appear to be facilitated by the presence of AT-rich sequences and absence of GC base pairs.

Phosphate contacts

In order to probe residues within the Fis HTH region for their roles in contacting specific phosphates, we used a strategy related in principle to that described in the missing base assay, only this time we utilized ethylated DNA. Ethylation interference assays were conducted on DNA fragments containing our high-affinity Fis binding site using various mutant Fis proteins and their results compared to that obtained with WT Fis. Of ten single alanine replacements within the Fis HTH region that were examined in this assay (N73A, Q74A, T75A, N84A, T87A, R89A, K90A, K91A, K93A and N98A), four (N84A, T87A, R89A, and K90A) yielded informative results.

Our results showed that ethylation of the phosphate 5' to the +4G (+4P) was well tolerated in the N84A Fis-DNA complex but not in the WT Fis-DNA complex (Fig. 5a). No other phosphates showed a similar pattern of relative tolerance to ethylation in the complex with N84A Fis. This indicates that the +4 phosphate is linked to the function of N84 in WT Fis, and suggests that this phosphate serves as a contact site for N84 (Fig. 5c). Similarly, ethylation of the phosphate 5' to -7G (-7P) showed considerably less interference to binding by R89A Fis than by WT Fis, suggesting that this phosphate is linked to the role of R89 and is a likely contact site by this residue (Fig. 5a and c).

The T87A Fis mutant presented a more complex picture in these assays. Ethylation of the phosphates 5' to +3T (+3P) and +4G (+4P) was better tolerated in the T87A Fis-DNA complex compared to the WT Fis-DNA complex (Fig. 5a and b). A slight increase in tolerance to ethylation of -7P was also detected among DNA complexed with T87A than with WT Fis. This suggests that the loss of the T87 side chain results in the loss of several phosphate contacts, which is consistent with the severe reduction in Fis binding caused by the T87A mutation. Since the T87 side chain is near N84 (within 3 Å) but on opposite side of R89 in the recognition helix of the HTH region (Fig. 6b), then a role for T87 in phosphate contact would most likely involve +3P and/or +4P but not -7P (Fig. 5c). On the other hand, several ethylated phosphates in the flanking DNA region that seemed innocuous to WT Fis binding were seen to interfere with T87A Fis binding. These were the phosphates 5' to the bases at positions +10, +11, +12, +13 and +14 (Fig. 5b and c). This suggests that, in absence of the DNA contacts made by the T87 side chain, additional interactions between Fis and phosphates in the flanking region are required. The notion that T87A Fis relies on extended interactions with the DNA flanking

region is consistent with similar observations from the T87A Fis binding to depyrimidinated DNA (Fig. 4b).

Since the K90A Fis mutant bound very poorly to DNA even at μM Fis concentrations (Fig. 3),²⁰ we opted to examine the binding of K90A/WT Fis heterodimers in our ethylation interference assays. The results showed that DNA complexes made with the K90A/WT heterodimers were considerably more tolerant to ethylation of +3P and +4P compared to the complexes formed with WT Fis (Fig. 5b). This indicated that the role of the K90 residue is somehow linked to the roles of these phosphates in Fis-DNA binding and suggests that K90 may interact with either or both of these phosphates (Fig. 5c).

DISCUSSION

Interactions with bases

Results from missing base assays presented evidence in support of interactions between R85 and the -7G base and between T87 and the +3T and +2T bases (Fig. 6). Results from GEMSA were consistent with the notion that T87 interacts with the base pair at +3. Other support for some of these interactions came from docking models suggesting that the R85 and T87 side chains extend in the general direction of the -7 and +3 bases, respectively (Table 2),^{15; 27; 28} from the strong conservation of the -7G•C and +3T•A base pairs,^{4; 17; 31} and by the findings that the -7G•C and +3T•A base pairs and the R85 and T87 Fis residues are all salient contributors to the high Fis-DNA binding affinity.^{18; 20; 21; 32}

Given the short side chain of T87, interactions with the +3 base may be mediated by a water bridge. Water mediated interactions have been known to play important roles in other complexes with DNA such as those involving the Trp repressor, the lambda repressor, and the CAP-cAMP complex.^{33; 34; 35; 36} Yet, while base pair substitutions of +3T•A for +3A•T or +3G•C cause severe (>900-fold) reductions in Fis binding, a substitution to +3C•G only caused about a 3-fold reduction in binding.²¹ This suggests that Fis may also be able to interact with the +3C•G base pair. Using the missing base assay, we observed that the guanine in this base pair (and not the cytosine) was required for WT Fis binding (unpublished observations). Thus, it seems as if Fis has the flexibility to interact with either a T•A or a C•G bp at this position by primarily contacting the thymine or the guanine in these base pairs. Both of these bases place an oxygen in the major groove (thymine O⁴ and guanine O⁶) that may serve as a hydrogen bond acceptor. Thus, it is possible that T87 or another Fis residue may interact with the +3C•G base pair.

The link between the +2T base and the T87 residue is not clear (Fig. 2c). Replacement of +2T•A with any other base pair has negligible effects on Fis binding, suggesting that this base pair, in of itself, is not used as a specific contact site.²¹ However, +2T contributes to the AT-richness in the central region of the binding site, which facilitates the flexibility required for Fis-induced DNA bending.²¹ Results from both the missing base and ethylation interference assays indicate that stable DNA binding by T87A Fis requires extensive interactions with the flanking DNA region involving the phosphates from +10 to +14 (Fig. 5). The preference by T87A Fis for the presence of A•T base pairs and for the absence of G•C base pairs in the flanking region (Fig. 4) suggests that AT-rich flanking sequences provide the necessary DNA flexibility to facilitate interactions in the flanking region. In this situation, the structural contribution by +2T may be dispensable.

Interactions with phosphates

We identified 14 phosphates along the DNA backbone of a high-affinity binding site that interfered with WT Fis binding when ethylated, demonstrating a prominent role played by

phosphate contacts in stabilizing Fis-DNA complexes. This observation complements our previous finding that Fis relies on a surprisingly small number of base contacts at a high-affinity DNA binding site.²¹ The critical phosphates are those at the 5' positions of the bases located at -8, -7, -6, +1, +2, +3, and +4 in each DNA strand relative to the central bp of the Fis binding site (Fig. 6a). Hence, the core Fis binding site should minimally include a 17 bp DNA region extending from -8 to +8.

The role of N84 was linked to that of the phosphate 5' to the +4 base (+4P), strongly suggesting that N84 interacts with this phosphate (Fig. 6). This result is in good agreement with predictions made from three Fis-DNA docking models, which consistently place the N84 side chain in contact with +4P (Table 2).^{15; 27; 28} While the same docking models place the R89 side chain in contact with a range of bases or phosphates at positions from -5 to -8 (Table 2), our results specifically linked the role of R89 to that of the phosphate 5' to the base at -7 (Fig. 6). We found no evidence for interactions between R89 and other phosphates or with the base pair at -7. It is possible that R89 may contact other positions at other DNA binding sites, such as the -5T and -6G bases in the *hin* distal Fis binding site (Table 2). However, we previously showed that -6G can be replaced with T or C with no decrease on Fis binding and that -5T can be replaced with A or G with only about a 2 to 6-fold decrease in binding.²¹ These effects are small compared to those of the base pairs at -7 (>2,000-fold) and +3 (>900-fold), which appear to serve as base contacts. Thus, it seems unlikely that the bases -5T and -6G generally act as important contact sites.

The role of the T87 Fis residue appears to be more complex. In addition to its previous connection to the roles of the base pair at +3, our results from ethylation interference assays linked the role of T87 with those of +4P, +3P, and to a smaller extent -7P. This is in agreement with Fis DNA docking models, which suggest an interaction between T87 and +3P, with some of the models also suggesting interactions with +4P or +2P (Table 2). If we dock the Fis recognition helix onto a DNA carrying our high affinity Fis site such that R85, R89, and N84 form hydrogen bonds with -7G, -7P, and +4P, respectively, we find that the T87 side chain comes within hydrogen bonding distance to both the +3 and +4 phosphates, in good agreement with our observations (Fig. 6b). It is possible that T87 contacts either of these phosphates on each half of the binding site or that sequential contacts with these two phosphates are important in the process of Fis-DNA binding and bending. However, we cannot rule out the possibility that the alanine replacement of threonine 87 alters the positioning of nearby side chains (e.g. N84 and K90) such that they are unable to contact either or both +3P and +4P. The -7 phosphate is too far from T87 (>14 Å) in this model for interactions to be feasible between T87 and -7P. Thus the link between the roles of T87A and -7P must not be direct.

The DNA complex formed with T87A Fis requires contacts with the phosphates in the flanking region at +10, +11, +12, +13, and +14. This implies that considerable structural distortions are made in the flanking DNA regions in order to facilitate such interactions in this complex. We previously found that the DNA-binding contribution of the R89 residue depends on the degree of DNA bending that occurs in the flanking region and is largely dispensable when the flanking DNA is highly bent.²⁰ This may be related to our finding that ethylation of -7P is slightly better tolerated when extensive interactions occur between the flanking DNA region and T87A Fis.

Docking models have placed K90 in contact with +1P, +2P, +3P or +4G, which underscores the flexibility of this long side chain and its potential to make different contacts in the DNA region from +1 to +4. Our results with ethylation interference assays linked the role of K90 with those of the phosphates +3P and +4P, suggesting that the K90 side chain may somehow interact with these two phosphates. Our docking of the recognition helix onto a high affinity Fis binding sequence suggests that the K90 side chain comes within hydrogen bonding distance

to both the +3 phosphate and the T87 side chain (Fig. 6b). A hydrogen bond between the K90 and T87 side chains could stabilize their positions to facilitate the interactions between T87 and both +3P and +4P and between K90 and +3P. In this case, the absence of K90 could be envisioned to disrupt all contacts with the +3 and +4 phosphates.

Interactions with the flanking DNA region

Our assays did not show how WT Fis interacts with the flanking DNA region beyond position -8. However, our results using the T87A Fis mutant revealed five additional phosphates in the flanking region from +10 to +14 that may serve as Fis contact sites. This raises the total number of possible phosphate contacts to 24. This represents a considerably larger number of Fis-DNA phosphate interactions in the bound complex than those reported for many other HTH DNA binding proteins such as the catabolite activator protein (CAP),^{24; 37} Tet repressor,³⁸ 434 cro repressor,³⁹ λ repressor,⁴⁰ and gamma delta resolvase,⁴¹ in which a range of between 8 to 14 phosphate contacts are identified in the bound complex. Whereas WT Fis also may be capable of interacting with phosphates in the flanking region from +10 to +14, such interactions may not all be essential or may have small effects on the binding affinity when an optimal number of contacts are made in the core-binding region from -8 to +8. Hence, the loss of any one of these phosphate contacts (e.g. by ethylation) may not sufficiently impact the WT Fis-binding affinity to allow detection in our assays.

Previous DNA cleavage studies using Fis conjugates to 1,10 phenanthroline copper (OP) demonstrated that Fis comes in contact with the DNA flanking the core Fis binding site, depending on the degree of Fis-induced DNA bending.^{17; 19} When OP was linked to residues 71 and 73 (immediately preceding the Fis HTH region), DNA cleavage could be observed at several different Fis binding sites in the flanking region from +8 to +14, suggesting that these residues come sufficiently close to this flanking region to permit contacts with its backbone phosphates. This is in good agreement with our results indicating that multiple phosphate contacts are made between T87A Fis in this very same DNA region. Replacements of R71 and N73 with cysteine and alanine, respectively, result in a decrease in Fis-induced DNA bending, which is consistent with their proposed roles in interacting with the DNA flanking regions.^{17; 42} Because of their positions on the Fis protein, residues Q74, T75, and R76 also have the potential to interact with phosphates in the flanking DNA region. Replacements of these residues to alanine were shown to have moderate effects (about 2 to 10-fold) on the Fis binding affinity to different DNA sequences.²⁰ Since binding by T87A Fis strongly depends on phosphate contacts in the flanking region (enough to allow their detection by ethylation interference), it may be possible to pinpoint potential phosphate interactions made with R71, N73, Q74, T75, and R76 by conducting ethylation interference assays with double Fis mutants carrying T87A together with an alanine replacement of one of these other residues.

Fis-induced DNA bending is facilitated by AT-rich sequences at positions -2 to +2 of the core Fis binding site and at positions -8 and -9.^{17; 21} Our results with T87A Fis showed that interactions with the flanking DNA are also facilitated by AT rich sequences and hindered by GC-rich sequences in the region from -9 to -19. Nucleotide substitution studies conducted in the *tyrT* promoter region, which contains four Fis binding sites, showed that the presence of the 2-amino group of guanine that resides in the minor groove, decreases the DNA intrinsic curvature and flexibility.⁴³ Replacements of guanines for inosines (which lack the 2-amino group) increased the intrinsic DNA curvature as well as the Fis-induced DNA bends, and resulted in a measurable increase in the Fis binding affinity. Conversely, replacements of adenines with 2, 6-diaminopurines (which contain a 2-amino group) decreased the intrinsic DNA curvature, the Fis-induced DNA bends, and the binding affinity. Thus, the absence of the 2-amino group in adenine bases seems necessary to allow the conformational freedom of AT-rich sequences to increase the DNA flexibility required for Fis-induced bending.

Implications for non-specific DNA binding

Whereas specific Fis-DNA interactions have been seen to occur in vitro with K_d values of about 1 to 4 nM, non-specific DNA interactions occur with K_d values in the μ M concentration range.²⁰ Therefore the binding of T87A Fis to DNA, which occurs in the μ M concentration range, may provide insight into the manner in which Fis binds non-specifically with DNA. As with the T87A Fis-DNA complex, non-specific Fis-DNA binding likely involves numerous phosphate contacts within a region extending from the center of the binding site to as far as 14 base pairs in either direction. The full extent of phosphate interactions will depend on the local DNA flexibility, which is in turn affected by the AT-richness of binding region. Indeed, in recent work employing chromatin immunoprecipitation combined with high density microarrays (ChIP-chip) to study the binding of three nucleoid-associated proteins across the *E. coli* genome in vivo, a large number of DNA binding positions was identified for Fis (>20,000) within AT-rich sequences.⁴⁴ The numerous phosphate interactions that occur within a 30 bp DNA region would require considerable DNA wrapping around the sides of Fis. Several Fis dimers binding DNA in phase may collaborate to further bend DNA forming microloops and altering the DNA topology and level of DNA compaction.^{45; 46; 47; 48}

MATERIALS AND METHODS

Chemicals, enzymes, and DNA

General chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Enzymes were purchased from New England Biolabs, Inc. or Roche Molecular Biochemicals. Formic acid (88%) was purchased from J. T. Baker. Ethylnitrosourea and piperidine were from Sigma-Aldrich. Radioisotope [α -³²P]dATP (3000 Ci/mmol) was from Amersham. Oligonucleotides were synthesized by the Center for Functional Genomics of The University at Albany, NY or by Integrated DNA Technology, Co. Purified wild type and mutant Fis proteins were made as previously described.²⁰ DNA plasmids used in this work are listed in Table 1 and were previously described.²¹

Gel electrophoretic mobility shift assay (GEMSA)

Plasmids pJS440, pJS453, and pRO436 were digested with BamHI and EcoRI to release a 43 bp DNA fragment that harbors a Fis binding site near its center. The DNA fragment was labeled with [α -³²P]dATP using the Klenow enzyme fill-in reaction, as described.⁴⁹ GEMSA was performed as previously described using approximately 1 fmol ³²P end-labeled DNA and various concentrations of WT or mutant Fis proteins.^{20; 21}

Formation of Fis heterodimers

Previous studies demonstrated that a dynamic subunit exchange between subunits of different Fis dimers rapidly occurs in solution at 37°C.²⁹ Using similar conditions, we combined an excess of purified R85A Fis with WT Fis (using a 33:1 R85A/WT Fis ratio). At equilibrium, the mixture becomes mostly populated with R85A Fis homodimers, which do not exhibit significant DNA binding at Fis concentrations below 4 μ M. However, most of the WT Fis subunits are expected to dimerize with R85A subunits under these conditions, such that the DNA binding activity in this mixture is almost entirely caused by the R85A/WT heterodimers. Heterodimers of K90A/WT Fis were similarly formed.

DNA missing-base assay

A HindIII-BamHI 75-bp DNA fragment containing a high-affinity Fis binding site (5'-GCTCAAATTTTGAGC-3') was obtained from pJS440. A single DNA strand was end-labeled with ³²P at either the HindIII or BamHI site by first cleaving pJS440 with one of these restriction enzymes and filling the ends with Klenow enzyme in the presence of [α -³²P]dATP, as

described.⁴⁹ Labeled DNA fragments were purified by 8% polyacrylamide gel electrophoresis and eluted using the crush and soak method.⁴⁹ DNA carrying random apurinic or apyrimidinic (AP) sites were made as described by Brunelle and Schleif.⁵⁰ To generate apurinic sites, about 5×10^5 cpm of ^{32}P -end labeled DNA was mixed with 3 μg sonicated salmon sperm DNA in 15 μl of 0.4% (v/v) formic acid and incubated at 37°C for 10 min. The DNA was then precipitated twice in the presence of 83 mM sodium acetate and 70% ethanol, rinsed with 70 % ethanol, dried under vacuum, and resuspended in 20 μl H₂O. To generate apyrimidinic sites, the ^{32}P -end labeled DNA was mixed with 3 μg sonicated salmon sperm DNA in a 25 μl volume of 37% (vol/vol) hydrazine anhydrous and incubated at room temperature for 10 min. DNA was then precipitated by addition of 1 ml butanol, resuspended in 100 μl H₂O, and precipitated twice in the presence of 83 mM sodium acetate and 70% ethanol. The DNA pellet was rinsed with 70% ethanol, dried under vacuum, and resuspended in 20 μl H₂O. The resulting AP DNA was used in GEMSA in the presence of sufficient Fis to achieve about 40–50% binding. The bound and unbound DNA were excised from the gel, recovered using QIAEX®II Gel Extraction kit (QIAGEN Inc.), and subjected to DNA backbone cleavage by incubating at 90°C for 30 min in the presence of 10% (vol/vol) piperidine. The DNA was dried under vacuum, resuspended in 90% (vol/vol) formamide and 0.01% bromophenol blue, heated to 90 °C for 2 min, and electrophoretically separated in 12% polyacrylamide–8 M urea gels.

Ethylation interference assay

The ethylation interference assay was performed as described.^{38; 51} About 5×10^5 cpm of ^{32}P end-labeled DNA (75 bp in length) was mixed with 3 μg sonicated salmon sperm DNA in 100 μl of 50 mM sodium cacodylate and incubated at 50°C for 5 min. Upon addition of 100 μl of saturated ethylnitrosourea solution (in 95% ethanol), the mixture was incubated at 50°C for 45 minutes. The DNA was then precipitated twice in the presence of 83 mM sodium acetate and 70% ethanol, rinsed with 70% ethanol, dried under vacuum, and resuspended in 20 μl H₂O. The resulting ethylated DNA was used in GEMSA in the presence of sufficient Fis to achieve about 40–50% binding. The electrophoretically separated bound and unbound DNA was excised from the gel and recovered using QIAEX®II Gel Extraction kit (QIAGEN Inc.). The DNA was then resuspended in 20 μl sodium phosphate buffer (10 mM sodium phosphate, 1 mM EDTA, pH 7), followed by the addition of 2 μl of 1.5 M sodium hydroxide. The mixture was incubated at 90°C for 30 min and dialyzed in H₂O for another 30 min. The DNA cleavage products were then dried under vacuum, resuspended in 90% (vol/vol) formamide and 0.01% bromophenol blue, heated to 90 °C for 2 min, and electrophoretically separated in 12% polyacrylamide–8 M urea gels.

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Abbreviations

AP, apurinic or apyrimidinic sites; Fis, factor for inversion stimulation; GEMSA, gel electrophoretic mobility shift assay; HTH, helix-turn-helix DNA binding motif; NAP, nucleoid-associated protein; OP, 1,10 phenanthroline copper; WT, wild-type.

Figure 1.

The Fis protein structure. (a) Ribbon model representation of the homodimer crystal structure from residues 26 to 98 (Protein Data Bank accession 3FIS). Residues 1–25 are largely unresolved in this structure. The four α -helices (α -A, α -B, α -C, and α -D) of one Fis subunit are labeled in order from the N-terminal to the C-terminal region. (b) Enlarged view of the region containing helices α -B, α -C and α -D in one Fis subunit. The HTH DNA binding motif consists of helices α -C and α -D. The side chains of the five most important Fis residues involved in DNA binding are shown. Residues R85, T87, and K90 (labeled in magenta) are absolutely required for Fis binding to different Fis binding sites, whereas residues R89 and K91 (labeled in green) make variable contributions to the binding affinity at different binding sites.²⁰ Images were generated using Swiss-PDB viewer v. 3.9b2.

Figure 2.

DNA phosphates involved in Fis binding. (a) Results of ethylation interference assays are shown for both the top and bottom DNA strands as they appear in panel (b). Lanes M contain Maxam and Gilbert G+A DNA sequencing products, which migrate 1 nucleotide slightly faster than the cleaved ethylated phosphates due to the chemical removal of the corresponding nucleotides in the former. Lanes S contain the DNA cleavage products of ethylated DNA prior to Fis binding; lanes U and B contain DNA cleavage products from electrophoretically separated unbound and Fis-bound ethylated DNA, respectively. Vertical lines indicate the positions of the core Fis binding site. Arrows point to positions showing relatively less cleavage in the bound (B) lane versus unbound (U) lane. (b) DNA sequence indicating the phosphates

involved in Fis binding. The Fis core binding sequence is shown in capital letters, which are numbered from -7 to +7; flanking sequence is shown in lower case letters. Phosphates are represented by a lowercase letter p. Phosphates showing strong ethylation interference are indicated with filled circles and phosphates showing moderately strong ethylating interference are indicated with open circles. (c) Double helical DNA representation of the Fis-DNA binding sequence. Nucleotides with 5' phosphates causing ethylation interference of Fis binding are colored in red.

Figure 3.

Loss of contact assay. WT Fis and four Fis mutants (T87A, R89A, K90A, and K91A) were tested for their abilities to bind a high-affinity Fis binding site and two low-affinity Fis binding site variants (-3C/+3G and -7C/+7G) using a gel electrophoretic mobility shift assay (GEMSA). The core DNA sequences of the three binding sites are shown at the top and are numbered from -7 to +7. Nucleotides differing from the high affinity site are shown in bold letters. Fis protein concentrations used in the binding reactions are shown underneath their corresponding lanes. Note that WT Fis concentrations used to bind the high-affinity site are in nM, whereas all other concentrations are in μM. The positions of the free DNA and bound DNA are designated for the GEMSA results using WT Fis.

Figure 4.

DNA base interactions with R85A and T87A Fis residues. Missing base assay performed with (a) depurinated or (b) depyrimidinated 75-bp DNA fragments carrying the high affinity Fis binding sequence shown in panel (c). Lanes M in panels (a) and (b) contain the products of Maxam-Gilbert G+A and C+T sequencing reactions, respectively. The U and B lanes contain the piperidine cleavage products from the unbound and Fis-bound DNA fragments, respectively. The Fis protein used in the binding experiments is indicated above the corresponding set of lanes. A vertical line on the left of each gel image indicates the region spanning the 15-bp core binding sequence. The -8A, -7G, -2A, and -1A purines required for WT Fis binding are indicated in panel (a). The +2T and +3T pyrimidines required for WT Fis binding are indicated with arrowheads in panel (b). Filled circles indicate positions of cleavage products corresponding to T bases that are less intense in the T87A Fis-bound DNA compared

to the unbound DNA. Open circles indicate positions of cleavage products corresponding to C bases that are more intense in the T87A Fis-bound DNA compared to the unbound DNA. (c) Summary of results from missing contact experiments. The core Fis-DNA binding sequence is shown in capital letters and numbered from -7 to +7. Flanking DNA sequences are shown in lower case letters. Bold letters represent bases that are strongly required for binding by WT Fis. Vertical lines designate critical bases that are not required for binding by R85A or T87A mutant Fis proteins, and are thus potential base contacts for the for R85 or T87. Dotted lines over +2T denote a suspected indirect link to the role of T87. Filled circles indicate T bases seen in depyrimidination assays to be required for T87A Fis binding. Open circles indicate C bases that hinder T87A Fis binding.

Figure 5.

Probing DNA phosphate contacts. Results of ethylation interference assays are shown for both the (a) top or (b) bottom DNA strands as they appear in panel (c). The Fis proteins used are indicated above their corresponding lanes. Lanes M contain Maxam and Gilbert G+A DNA sequencing products; lanes S contain the DNA cleavage products of ethylated DNA prior to Fis binding; lanes U and B contain DNA cleavage products from electrophoretically separated unbound and Fis-bound ethylated DNA, respectively. Vertical lines indicate the positions of the core Fis binding site. Arrowheads point to positions showing relatively more cleavage in the bound (B) vs. unbound (U) fraction with a mutant Fis protein compared to that observed with WT Fis. (c) DNA sequence indicating the phosphates involved in Fis binding. The Fis core binding sequence is shown in capital letters, which are numbered from -7 to +7. Flanking sequence is shown in lower case letters. Phosphates are represented by a lowercase letter p. Phosphates showing strong ethylation interference to WT Fis binding are indicated with filled circles and phosphates showing moderately strong ethylating interference are indicated with open circles. Lines indicate suggested interactions between certain phosphates and the Fis N84, 4 T87, R89, and K90 side chains. Asterisks indicate phosphates required for T87A Fis binding.

Figure 6.

Major determinants in Fis-DNA binding. (a) Fis interactions with a high-affinity DNA binding site. The 15-bp core sequence is shown in capital letters and flanking sequences are shown in lower case letters. Boxed base pairs are highly conserved and were shown to be critical for WT Fis binding using nucleotide substitution studies.²¹ Bold letters represent bases shown to be required for WT Fis binding as observed from missing base assays. Phosphates that are strongly or moderately strongly required for WT Fis binding are indicated with filled or open circles, respectively. Interactions between Fis and DNA are represented by lines connecting a residue with the corresponding base or phosphate. Dotted lines between K90 and +4P represent a suspected indirect link. (b) Model representing critical interactions between Fis residues in the recognition helix of the HTH region (α -D helix) and DNA. Nucleotides -7G (red), +4G (purple), +3T (blue) and Fis side chains N84, R85, T87, R89, and K90 are labeled. Hydrogen bonds are represented with dotted lines.

Table 1

Plasmids used in this work.

Plasmids	Fis Binding Site [†]	Source
pJS440	5'-GATCTAGCTCAAATTTGAGCTATAGCT-3'	21
pJS453	5'-GATCTAGCTCCAATTGGAGCTATAGCT-3'	21
pRO436	5'-GATCTATCTCCAATTGGAGATATAGCT-3'	21

[†] Complementary oligonucleotides carrying the indicated sequences were annealed and cloned into the BglII and SacI sites of pCY4 to create the corresponding plasmids.

Table 2

Interactions predicted by Fis-DNA docking models

Fis residue	DNA contacts [†]		
	Model 1 [‡]	Model 2 [‡]	Model 3 [‡]
N84	+4P/+4P	+4P/+4G ; +5A	+4P/+4P
R85	-7G/-7G	-7G ; -8P; -9P/-8P; -9P	-7G/-8A
T87	+3P/+3P ; +2P	+3P/+3P	+3P/+4P
R89	-7P/-6P ; -5T; -6G	-7G ; -8P/-8P	-7G/-6G
K90	+3P ; +2P/+1P; +2P	+1P; +2P/+2P	+4G/+4G

[†] DNA contacts are numbered according to their positions on the Fis binding site in Fig. 2b. Putative contacts made on opposite DNA strands are separated by a slash (/) and multiple putative contacts made on the same DNA strand are separated by semicolons (;). The phosphates are those located 5' to the base position indicated. DNA contacts suggested from the present work are shown in bold.

[‡] Fis-DNA docking model 1 is according to Tzou and Hwang.²⁸ Model 2 is according to Sandman, et al.²⁷ Model 3 is according to Feng, et al.¹⁵ All three are modeled using the *hin* distal Fis binding site, which participates in the Hin-mediated DNA inversion process.⁵²